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DNA ADDUCTS AND CIGARETTE SMOKING

I. Background

Research on DNA adducts has followed the recent trend of research attempting to develop specific biomarkers of chemical exposure. Investigators have, for example, attempted to track cigarette smoking by using carboxyhemoglobin levels as a measure of carbon monoxide exposure; thiocyanate levels as a measure of hydrogen cyanide exposure; and cotinine measured in the urine, plasma or saliva as indicators of nicotine exposure. Recently, scientists have proposed that measurements of DNA adduct levels would indicate exposure to any chemical agent that modified cellular DNA.

The term adduct means chemical addition product and can be used to denote covalent bonding of chemical compounds to substances such as DNA. For example, there are studies reporting hemoglobin adducts in red blood cells. The utility of DNA adduct measurements resides in the general observation that most carcinogens appear to bind DNA, and in the theory that interactions between carcinogens and DNA are critical events in the initiation of carcinogenesis. Eventually, the goal of DNA adduct research may

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be to measure exposure to carcinogens and to estimate human cancer risk by measuring DNA adduct levels.

The evaluation of DNA adduct levels in human tissue was not feasible prior to 1980, because the available methodologies were not sensitive enough to isolate very low levels of adducted compounds. However, with the introduction of more sensitive analytical procedures, research in identifying minute levels of DNA adducts in animals increased in the early 1980's. Two notable assays, the enzyme linked immunoabsorbent assay (ELISA) and the ^{32}P-post labeling assay, have now allowed the isolation of extremely small amounts of adducted DNA in tissue. The ^{32}P-postlabeling allows the measurement of one DNA adduct to 10^8 nucleotides, and it benefits from radiolabeling after the tissue is removed from the organism.

Although both the ELISA and the ^{32}P-postlabeling techniques are equally sensitive, the ^{32}P-postlabeling assay represents a more efficient and easier approach to isolating DNA adducts (see figure 1 for isolation scheme). This assay was used in the Phillips study. Briefly, the DNA is treated with enzymes to break down the DNA into its basic building blocks called nucleotides. Then the nucleotides are treated with an enzyme in order to attach a specific radioactive compound (^{32}P) to the nucleotides. Separation and

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resolution of normal nucleotides from adducted nucleotides (DNA adducts) is performed by thin layer chromatography. This technique permits the mapping of the nucleotides on the chromatographic film. The "spots" on the film are identified and quantified utilizing autoradiographic procedures, which result in the development of a "fingerprint" pattern. Normal DNA has one pattern, or "fingerprint" on the film, and DNA adducts have another.

This ^{32}P-postlabeling procedure becomes important because of references in the Phillips paper and other publications to the adducted DNA appearing at a specific location on the chromatograph known as adduct 1. Adduct 1 has been associated with polycyclic aromatic hydrocarbon exposures.

In 1982 F. Perera reported several studies of DNA adduct isolation from animal tissues. After injecting animals with benzo[a]pyrene, Perera found elevated levels of DNA adducts in mouse lung tissue. These results were not supported by other animal and human research. For example, an American Health Foundation study of dogs exposed to cigarette smoke for 20 months found no significant difference in benzo[a]pyrene-DNA adduct levels between exposed and control animals. Another study conducted by Perera on human lungs failed to support her animal results. She studied DNA adducts from lung tissue of 27 patients with various smoking

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histories. She concluded that the benzo[a]pyrene-DNA adduct levels in smokers were not significantly different from nonsmokers, and she stated "we were not able to correlate occurrence of adduct levels in smokers with their level of cigarette smoking."

In 1986, Everson identified DNA adducts from human placental tissue utilizing ^{32}P-postlabeling. He demonstrated at best a weak association between smoking and the levels of DNA adducts in the placenta of smoking mothers. The association was not statistically significant. He conceded, however, that the assay might have detected DNA damage unrelated to cigarette smoking, since his controls also had elevated DNA adduct levels.

Interestingly, he observed the presence of adduct 1 in the DNA of smokers' placental tissue. As previously mentioned, adduct 1 has been associated with exposure to aromatic hydrocarbons which are found in cigarette smoke and are ubiquitous in the environment. Dr. Everson did not attribute the finding of adduct 1 specifically to the hydrocarbons found in cigarette smoke. Instead, he implied that adduct 1 might have been the result of a more general environmental exposure to hydrocarbons.

In 1987, Perera studied the hemoglobin adduct levels of 4-aminobiphenyl (4-aminobiphenyl has been shown to be in mainstream

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smoke). Perera reported a marginally significant increase in the levels of 4-aminobiphenyl hemoglobin adducts in smokers versus nonsmokers, but her evidence was not convincing since the number of subjects in her study was small and she was dealing with a surrogate adduct, i.e., an adduct isolated from the peripheral blood whose relevance to lung DNA adducts is questionable.

Other studies have reported DNA adducts in human or animal tissues exposed to cigarette smoke or its components. Many of these studies have been contradictory or preliminary in nature, and the relevance of some of the findings to human lung cancer is unknown. The science of DNA adduct characterization is at the cutting edge of basic research in biology. Since the biological significance of DNA adducts is not known, validation of the relationship of DNA adduct measurements to carcinogenesis will require thorough development of chronic animal models.

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B. Critique of Phillips paper

In 1988, Phillips et al published a letter to the editor in Nature which claimed to have found for the first time a linear relationship between cigarette smoking and the presence of DNA adducts in human lung . The popular press seized on this study as final proof of the mechanism by which smoking causes lung cancer, but that interpretation is flawed. The study does not show the mechanism by which smoking causes lung cancer. In fact, it does not even establish that the DNA adducts were caused by smoking.

Specific weaknesses of the study include the following.

1) There was an unequal number of smokers and nonsmokers in the study. The tissue levels of DNA adducts were analyzed for only 5 nonsmokers as compared to 17 smokers. This skewed sampling indicates a poorly designed and uncontrolled experiment.

2) There was an unusual statistical approach used for reporting DNA adduct levels in this study. Phillips reported the mean plus or minus a range of data which is difficult to interpret. It is better to report the mean plus or minus a standard deviation which gives a clearer measure of variability.

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3) A replot of the linear regression analysis of the Phillips data shows that it was done correctly. However, a simple Students t-test of differences between the mean DNA adduct levels of smokers and nonsmokers showed that they were not significantly different. This was not mentioned by Phillips in his report.

4) Because of limitations of the assay used, Phillips could not provide a chemical identification of a specific adduct. In other words, he could not show that the DNA adducts that he found were associated with any tobacco smoke components such as benzo[a]pyrene, 4-aminobiphenyl, a nitrosamine, etc. He could only report some "nonspecific" DNA adducts. In fact it has been shown by other investigators that alleged carcinogens in mainstream smoke have not shown the same chromatographic "fingerprint" as the Phillips DNA adduct.

5) The relationship between elevated DNA adduct levels and cigarette smoke is unclear. Phillips' and other studies' data show that elevated adduct levels 1) are caused by agents other than cigarette smoke, and 2) can occur in the absence of any known cigarette smoke exposure. For example, the nonsmokers in Phillips' study had elevated adduct levels, and five of the smokers had levels equivalent to or lower than the nonsmokers. In fact, the lowest

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level was found in a smoker and the next lowest level in a former smoker.

6) Analysis of lung tissue without fractionation or separation of the tissue into specific cell types to measure only the DNA adduct levels in "stem" cells is inappropriate. Lung cancer is thought to result from the transformation of stem cells, which comprise an extremely small percentage of all cells in lung tissue. The measurement of DNA adducts in general lung tissue does not yield data on what if any DNA adducts are present in the important stem cells. DNA adducts in non-stem cells would not necessarily have any relevance to lung cancer risk or causation.

7) The problems with measuring DNA adduct levels in tissues containing many different types of cells are illustrated by the great variation of DNA adduct formation in different animal cell populations. Belinsky found adduct levels to be significantly different in six different lung cell types isolated from Fischer 344 rat lung tissue.

8) The authors concede that they took no account of occupation, diet, socioeconomic group or environment in interpreting their findings. This is a crucial flaw in the study, since each of these factors could have significantly affected the

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patients' exposure to agents that lead to DNA adduct formation. For example, this study was conducted by researchers located in both London and Heslington. There are no data regarding the source of the patient population. Urban and rural residents may have vastly different DNA adduct levels.

9) Another important question is the appropriateness of combining male and female data. The mean DNA adduct level for current women smokers (9.4) was lower than that for men smokers (12.7). Clinical research and toxicological studies rarely combine male and female results.

10) All of the women in this study were 48 years of age or older. Dr. Joaquin Lehr of the University of Texas at Galveston has shown that steroid treatment stimulates DNA adduct formation. If any or all of the females in this study were receiving post-menopausal steroid replacement therapy (estrogen), such treatment could seriously confound the results.

11) The Phillips study utilized lung tissue from individuals ill with lung cancer. No information is given regarding chemotherapy, radiotherapy or other treatment these individuals may have received. The question remains whether the diseased state or intervening treatment of these individuals affected their immune

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function and DNA repair mechanisms, thereby influencing their levels of DNA adducts. For example, DNA adduct levels have been measured after cis-platinum therapy for cancer.

12) Dr. Phillips claimed a linear relationship between the number of cigarettes smoked and DNA adduct levels in the lung. This linear relationship is only a correlation, and data regarding tobacco specific DNA adducts in lung tissue are needed before linearity between number of cigarettes smoked and DNA adducts found in the lung as a result of cigarette smoking can be concluded.

13) Many inappropriate assumptions were made with regard to smoking history, i.e., the authors assumed that the subjects began smoking at 18 years of age. In addition, the authors used questionnaires to obtain smoking data as opposed to some other measurement i.e., cotinine, carboxyhemoglobin, etc. These assumptions can lead to large errors in smoking history especially since Phillips uses the smoking history data for plotting his linear regression data and bases his entire conclusion (correlation) on the weak smoking history data.

The subject of DNA adducts is not just of academic interest. It has already surfaced in cigarette products liability litigation in the United States. Dr. H.K. Ginzel, who appeared as

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an expert witness for the plaintiffs in the Horton case in Mississippi and in the Girton case in Pennsylvania, relied on DNA adduct research to conclude that inhaled cigarette smoke constituents were responsible for damaging DNA and ultimately causing lung cancer.

In conclusion, the Phillips paper represents a very preliminary pilot-type study on the relationship of DNA adducts in lung tissue in smokers and the number of cigarette smoked per day. The study was poorly controlled, and contained many serious scientific flaws including: poor or incomplete smoking histories, unequal group sizes, no justification for combining data from male and female subjects, less than rigorous statistical analysis, lack of attention to specific pulmonary cell analysis, disregard for important factors such as the effects of steroid replacement therapy in post-menopausal women, ongoing or prior chemotherapy, prior radiation treatment, occupational history, age and nutrition.

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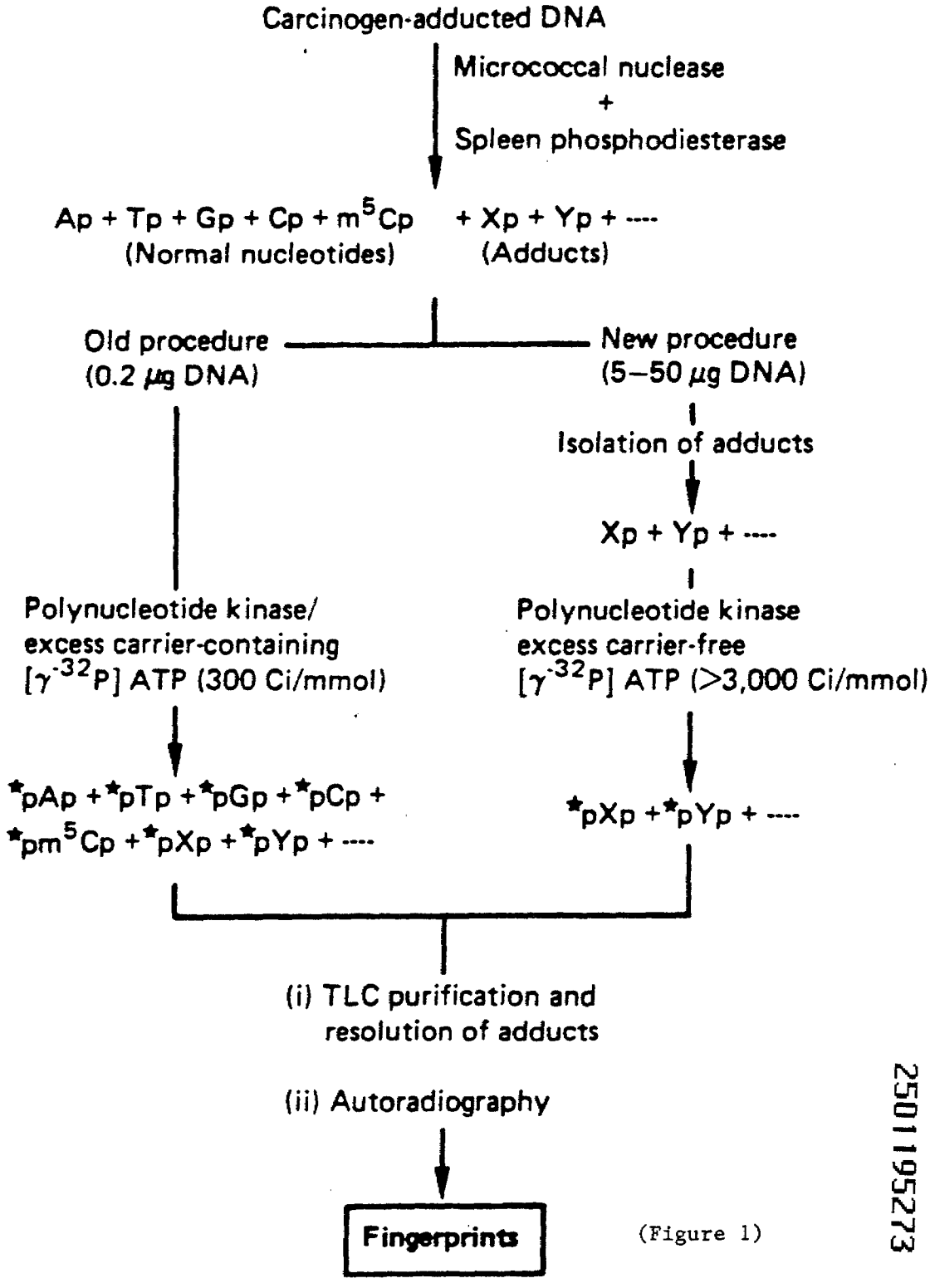
^{32}P-Adduct Assay



(Figure 1)

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